An *Arabidopsis* Rhomboid homolog is an intramembrane protease in plants

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Abstract Regulated intramembrane proteolysis (RIP) is a fundamental mechanism for controlling a wide range of cellular functions. The *Drosophila* protein Rhomboid-1 (Rho-1) is an intramembrane serine protease that cleaves epidermal growth factor receptor (EGFR) ligands to release active growth factors. Despite differences in the primary structure of Rhomboid proteins, the proteolytic activity and substrate specificity of these enzymes has been conserved in diverse organisms. Here, we show that an *Arabidopsis* Rhomboid protein AtRBL2 has proteolytic activity and substrate specificity. AtRBL2 cleaved the *Drosophila* ligands Spitz and Keren, but not similar proteins like TGFα, when expressed in mammalian cells, leading to the release of soluble ligands into the medium. These studies provide the first evidence that the determinants of RIP are present in plants.

Keywords: Intramembrane proteolysis; Rhomboid; Serine protease; Epidermal growth factor; Spitz; *Arabidopsis*

1. Introduction

Intercellular communication is a fundamental mechanism for coordinating the development of complex body forms that characterize multicellular organisms. Intercellular signaling also plays a key role in controlling homeostatic processes. One of the major mechanisms for this cell-to-cell communication is the transmission of signals through ligand-receptor interactions, such as growth factors and their receptors in animals.

Regulated intramembrane proteolysis (RIP) is one recently-discovered mechanism for controlling the production of signaling molecules [1]. In RIP, intramembrane-cleaving proteases (I-CLiPs) control the release of membrane-anchored proteins such as epidermal growth factors (EGFs) or transcription factors, by cleaving them in the plane of cellular membranes. There are at least four families of I-CLiPs known to be involved in RIP [2]. Presenilin-1, a member of Presenilin family, cleaves Notch to promote Notch signaling [3], as well as other proteins. Site2 protease, a member of S2P family, cleaves the SREBP transcription factors to regulate cholesterol biosynthesis, and ATF6 to signal the unfolded protein response [4]. Signal peptide peptidase (SPP) processes signal peptides, including cell surface histocompatibility antigen (HLA)-E epitopes in humans; the HLA-E epitope-containing fragment is subsequently released from the lipid bilayer [5].

The fourth member of this set of regulators of RIP is the recently discovered Rhomboid family of serine protease [6,7]. When the *Drosophila* EGF ligand Spitz, a type-1 membrane protein anchored to the ER, is recruited to the Golgi apparatus by Star, it is cleaved by Rhomboid-1 (Rho-1), producing a secreted form of Spitz that is able to activate EGF receptors (EGFRs) in adjacent cells [6]. Rhomboid proteases have substrate specificities; Rho-1 processes other *Drosophila* EGF ligands such as Keren and Gurken [8] but not TGFα, despite the fact that TGFα has type-1 membrane topology and an EGF domain [9].

The structure and substrate specificities of the Rhomboid family is widely conserved throughout the prokaryotic and eukaryotic kingdoms [7,9,10]. On the other hand, differences between the enzymes have been found. For example, many organisms contain Rhomboid proteins that contain all features known to be required for activity, yet do not process Spitz, Keren or Gurken, and probably thus have different substrate specificity.

Plants also have Rhomboid genes [10], but their function has not yet been characterized. In fact, there is no direct evidence that RIP exists in the plant kingdom. To understand the molecular properties of these proteins in plants, we isolated two Rhomboid-related genes from the genome of *Arabidopsis thaliana* and characterized their subcellular localization and protease activity. Like *Drosophila* Rho-1, both *Arabidopsis* Rhomboid proteins localized to the Golgi apparatus in plant cells. One of them, called AtRBL2, showed proteolytic activity and substrate specificity. This is the first evidence that proteolytic activity and substrate specificity of this family is also conserved in plants.

2. Materials and methods

2.1. Programs for alignment and prediction of transmembrane domains

Alignments were performed using the ClustalW algorithms [11] and modified by hand. Transmembrane domains were predicted according to the TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) and TMPred (http://www.ch.embnet.org/software/TMPRED_form.html) algorithms.

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Abbreviations: RIP, regulated intramembrane proteolysis; I-Clips, intramembrane-cleaving proteases; Rho, Rhomboid; AtRBL, *Arabidopsis thaliana* Rhomboid-like; EGF, epidermal growth factor; EGFR, EGF receptors; TMD, transmembrane domain; BFA, Brefeldin A

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2.2. cDNA cloning and RT-PCR

Total RNA from inflorescences of *A. thaliana* (wild type Col-0 ecotype) was isolated using RNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA). The first strand cDNAs were synthesized with the SuperScriptII First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). For the cloning of *AtRBL1* and *AtRBL2*, primers were designed according to the gene prediction model of AtGDB (http://www.plantgdb.org/AtGDB/index.php). Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the Accession Nos. AB195672 (AtRBL1) and AB195671 (AtRBL2), respectively.

For RT-PCR, cDNAs were synthesized from 0.9 μg of total RNA with SuperScriptII First-Strand Synthesis System. The tissues used were aerial part from seedlings 10 days after germination, root from seedlings 10 days after germination, rosette leaf, cauline leaf, stem, silique and inflorescence. Following gene-specific primers were used; AtRBL1-Forward (5′-GAGATCAAGGTGGTGAATCC-3′) and AtRBL1-Reverse (5′-ACACCTCGTGCTTATGAACC-3′). AtRBL2-Forward (5′-GGAGGAAAGTAGTACATGAACATCAAGG-3′) and AtRBL2-Reverse (5′-ATGAACAGAAGTGTGATCAGAGCAG C-3′). Primers for *ACT8* were followed as described in [12]. The number of PCR cycles was 35.

2.3. DNA construction

To make GFP-tagged constructs, G3GFP [13] was amplified by PCR and subcloned so as to replace the GUS gene of pBI121 (Clontech, USA) to generate p35SG3GFP. AtRBL1 and AtRBL2 were inserted between the CaMV 35S promoter and G3GFP using *Xba*1 restriction endonuclease to generate p35SAtRBL1-G3GFP and p35SAtRBL2-G3GFP, respectively. The *Hin*dII–*Eco*RI fragment of p35SAtRBL1-G3GFP and p35SAtRBL2-G3GFP were cloned into pBluescript-SKII+ (Toyobo, Japan) for transient expression. To make triple Haemagglutinin (HA)-tagged constructs, all Rhomboid genes were cloned into pcDNA3.1(-) (Invitrogen) by introducing unique restriction endonuclease sites during PCR from cDNA for AtRBL1 and AtRBL2. A triple HA tag was inserted after the initiator methionine of AtRBL1 and AtRBL2. All constructs were confirmed by se-

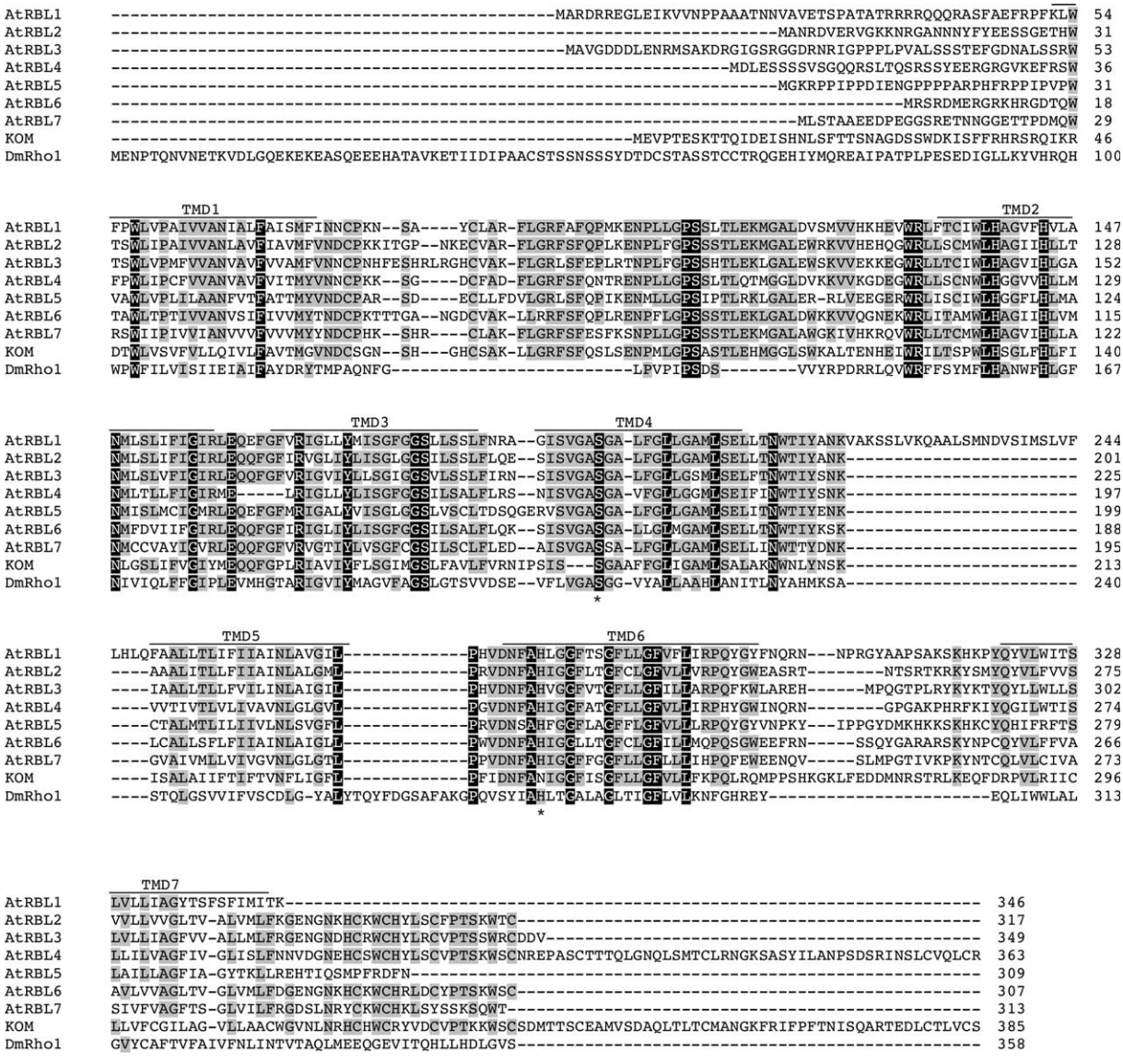

Fig. 1. A multiple sequence alignment of the *Drosophila* and *Arabidopsis* Rhomboid proteins. The sequence used were AtRBL1 (AGI code At2g29050), AtRBL2 (At1g63120), AtRBL3 (At5g07250), AtRBL4 (At3g53780), AtRBL5 (At1g52580), AtRBL6 (At1g12750), AtRBL7 (At4g23070), KOM (At1g77860), and DmRho-1 (Accession No. NM_079159). The amino acid residues conserved in all of the nine sequences were highlighted in black, conserved in at least five of the nine sequences were in gray. The approximate locations of the TMDs were denoted by black lines. The putative catalytic dyad residues, S (TMD4) and H (TMD6), were indicated by asterisks.

quence analysis. A triple HA-tagged Rhomboid-1 was described previously [6]. Spitz, Keren, and TGFα were tagged with GFP at their N termini as described previously [6–8].

2.4. Subcellular localization analysis

Transient expression of GFP-fused AtRBL proteins and Venus-SYP31 (a kind gift from T. Uemura) [14] in *Arabidopsis* Col-0 suspension culture cells [15] was performed by the method described previously [16]. Transformed protoplasts were incubated under gentle agitation at 23 °C for at least 8 h in the dark. Transformed cells were observed with a confocal laser microscope system (LSM510META, ZEISS, Jena, Germany) with the 488 nm line of an Ar/Kr laser. The fluorescence of GFP and Venus (a variant of YFP) [17] were distinguished using META system (ZEISS, Jena, Germany). For Brefeldin A (BFA) treatment, protoplasts were incubated in the culture medium that contains 100 μg/ml of BFA for 2 h in room temperature.

2.5. Ligand cleavage assay

The ligand cleavage assay was performed as described in detail previously [6–9]. Briefly, COS cells were transfected using Fugene6 (Roche) with 100 ng of each HA-tagged Rhomboid and 250 ng of other GFP-tagged constructs. Note that various concentrations of each HA-tagged Rhomboid were tested, but 100 ng proved optimal for all Rhomboids that showed activity. Star was cotransfected for the analysis of Spitz and Keren but not for TGFα. Empty vector pcDNA3.1 was added to adjust the total DNA content to 1 μg, which is necessary to normalize the transfection efficiency with each experiment. After 24 h, the media containing transfection reagents was replaced with serum-free media, and this media was conditioned for the following 24 h in the presence or absence of 20 μM Batimastat, a potent metalloprotease inhibitor. Ligands were detected in cells and media by Western analysis with anti-GFP polyclonal antiserum (kind gift of Dr. Rob Arkowitz).

3. Results and discussion

3.1. Sequence analysis of Arabidopsis Rhomboid genes

To study the molecular properties of Rhomboid proteins in plants, we conducted a computer-based search for Rhomboid-like genes in the genome of the thale cress plant, *A. thaliana* [18]. There were eight Rhomboid-like genes in the *Arabidopsis* genome (Fig. 1). Based on the BLAST results, we named the one that had the highest homology to *Drosophila* Rho-1 as AtRBL1. The other seven genes were AtRBL2 to AtRBL7 and KOMPEITO (KOM) (Kanaoka and Okada, unpublished data), respectively, with AtRBL2 showed the highest homology to AtRBL1 and KOM the lowest.

The TMHMM program predicted that each gene encoded a protein with the typical secondary structure of the eukaryotic Rhomboid family with seven transmembrane domains (TMDs) [19]. As is the case with this family [10], the overall sequence similarity was relatively low; AtRBL1 was 19.8% identical to Rho-1 and AtRBL2 was 17.7%. The region between the second TMD and sixth TMD showed the highest degree of sequence conservation with *Drosophila* Rho-1 (47% identical to AtRBL1 and 52% to AtRBL2), while the N-termini and the C-termini were more divergent. Despite the sequence diversity between *Drosophila* and *Arabidopsis* Rhomboid proteins, the Ser residue in the fourth TMD and the His in the sixth TMD, which formed the putative catalytic dyad found in all of Rhomboid proteins with proteolytic activity against *Drosophila* ligand Spitz [20], were conserved in AtRBL1 to AtRBL7, but the Asn may be replaced by His in KOM (Fig. 1). Moreover, AtBDL1 to AtRBL7 contained the conserved GASG motif surrounding the active serine [7].

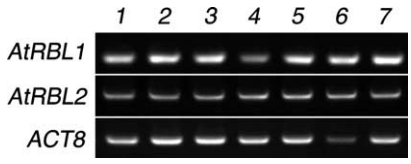

Fig. 2. Expression pattern of *AtRBL*1 and *AtRBL2*. Total RNA was extracted from aerial part from seedlings 10 days after germination, root from seedlings 10 days after germination, rosette leaf, cauline leaf, stem, silique and inflorescence (lanes 1–7, respectively). *ACT8* was used to normalize the RT-PCR.

3.2. Expression patterns and subcellular localization of AtRB1 and AtRBL2

To start addressing their biological functions, we cloned and investigated the expression of the two most Rho-1 like proteins, *AtRBL1* and *AtABL2*, by RT-PCR (Fig. 2). Both genes were expressed in all tissues tested, including roots, aerial parts of seedlings, rosette leaves, stems and flowers, indicating that these genes have a function in plants.

Many eukaryotic Rhomboid proteins are localized within the Golgi apparatus [6–8,21]. Spitz, a major substrate of Rho-1 protease, is anchored in the endoplasmic reticulum (ER) until Star chaperones it to the Golgi apparatus, where Rho-1 cleaves Spitz. To reveal the localization of AtRBL1 and AtRBL2, we fused each protein with the Green Fluorescent Protein (GFP) and investigated the subcellular localization in protoplasts prepared from *Arabidopsis* suspension culture cells (Fig. 3). The AtRBL1-GFP fusion protein accumulated in dot-like structures in the cytosol (Fig. 3A). This pattern significantly overlapped with Venus-SYP31, a cis-Golgi marker [14] (Fig. 3A–C). Moreover, when these cells were treated with Brefeldin A (BFA), which inhibits the activity of the ARF GTPase and inhibits vesicle transport from ER to the Golgi [22], most of these dots disappeared and the fluorescence profile was changed to one typical for the ER pattern (Fig. 3G-I). The same results were obtained when AtRBL2-GFP was tested (Fig. 3D-F, J-L). These data indicate that AtRBL1 and AtRBL2 are localized to the Golgi apparatus in plant cells.

While some Rhomboid proteins are imported into mitochondria where they function in membrane fusion [23], most localize to the Golgi apparatus and function in the secretory pathway. The observation that AtRBL1 and AtRBL2 concentrated in the Golgi apparatus thus suggests that AtRBL1 and AtRBL2 might function in the secretory pathway like Rho-1.

3.3. Proteolytic activity of AtRBL1 and AtRBL2

Rhomboid is a member of the serine protease family. They are present in most sequenced genomes of archea, bacteria and eukaryotes [10]. Members of this family from diverse organisms, including *Drosophila*, human and bacteria, have the ability to cleave specifically *Drosophila* substrates, including Spitz, Keren and Gurken [7,9,24].

To examine whether *Arabidopsis* Rhomboid proteins have similar proteolytic activity, we tested the ability of AtRBL1 and AtRBL2 to cleave two *Drosophila* transmembrane ligands. The cleavage of Spitz and Keren can be assayed in a mammalian cell transfection system [6–8]. When Spitz was cotransfected with AtRBL1 in the presence of Star, AtRBL1 could not cleave either of the two ligands (Fig. 4A, lane 5; B, lane

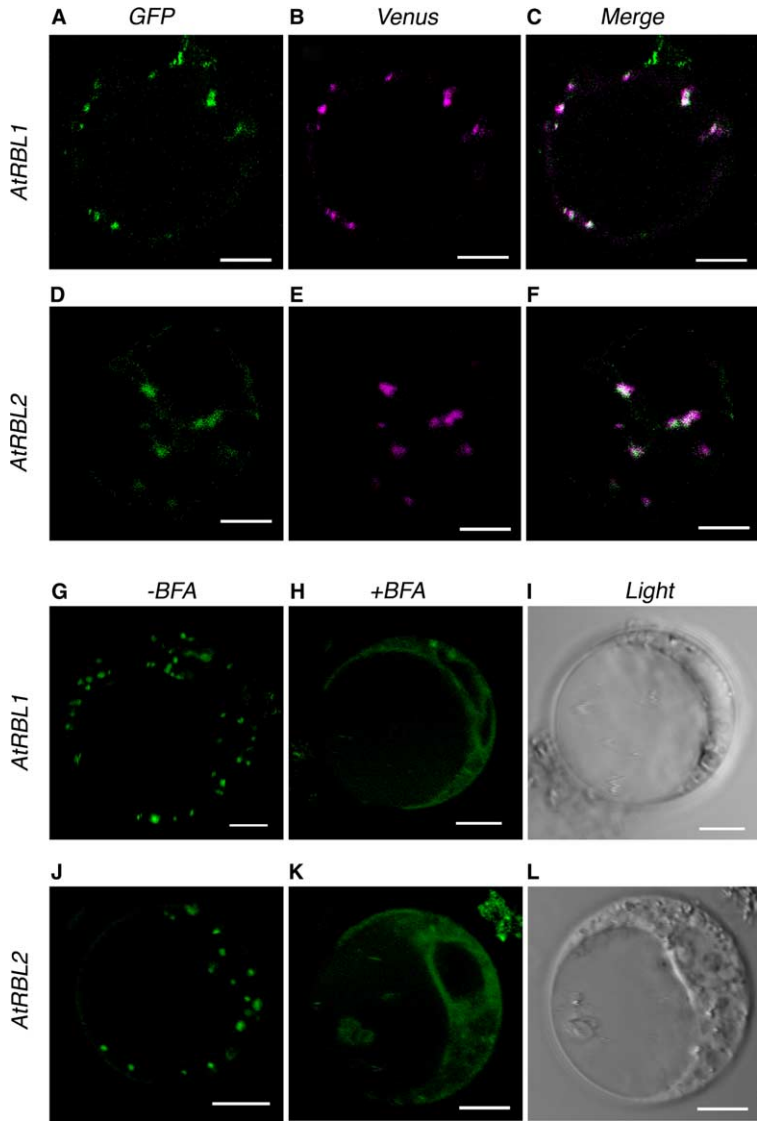

Fig. 3. Subcellular localization of AtRBL1 and AtRBL2. Protoplasts prepared from *Arabidopsis* suspension cells were transformed with AtRBL1-GFP (A–C, G–I) or AtRBL2-GFP (D–F, J–L). For the co-transformation with Venus-SYP31, Green (A,D), Purple (B,E) and White (C,F) colors show fluorescence from GFP, Venus and their combined images, respectively. Cells were treated with (H,K) or without (G,J) BFA. (I) and (L) are the Nomarski images of (H) and (K), respectively. Bar indicates 20 μm.

4), in spite of the sequence similarity of Rho-1 and AtRBL1 (Fig. 1). On the other hand, when AtRBL2 was cotransfected with Spitz and Star, the truncated form of Spitz was secreted from the cell to the media (Fig. 3A, lane 4). This indicates that Spitz was cleaved by AtRBL2, producing a protein of the same size as that produced by Rho-1 (Fig. 4A, lane 3). AtRBL2 also cleaved Keren in the same manner (Fig. 3B, lane 3). These findings are the first evidence that a plant Rhomboid protein has proteolytic activity against transmembrane ligands, despite the striking sequence divergence between *Drosophila* and *Arabidopsis* Rhomboid proteins (Fig. 1). Since Spitz was secreted, it is likely that the reaction took place in the Golgi apparatus where AtRBL2 is localized, since Spitz cleaved in the ER would not be secreted from the cell [8].

3.4. Substrate specificity in AtRBL1 and AtRBL2

One of the characteristics of the Rhomboid protease family is that they display strong substrate specificity, and for example, do not cleave even similar proteins such as TGFα, the human homolog of Spitz [9,25]. To determine whether *Arabidpsis* Rhomboid proteins also share this substrate specificity, we tested the ability of AtRBL1 and AtRBL2 to cleave TGFα using our transfection assay (Fig. 5). As expected, TGF was cleaved well by cellular metalloproteases (Fig. 5, lane 1) but not by *Drosophila* Rho-1 (Fig. 5, lane 3), or AtRBL1 and AtRBL2 (Fig. 5, lanes 5 and 4, respectively). Since both *Drosophila* Rho-1 and AtBDL2 could cleave Spitz but not for TGF, these results suggest that AtRBL2 is not a nonspecific protease but rather has the same selectivity as *Drosophila* Rho-1.

Our results showed that AtRBL2 has protein cleavage activity, but we did not show the activity of AtRBL1. AtRBL1 may have proteolytic activity on unknown substrates, and different function from those of AtRBL2. Several vertebrate Rhomboid proteins have been shown to have their own specific function [21,26]. There remains, however, a possibility that AtRBL1 protein was not adequately folded in the animal cells. Further

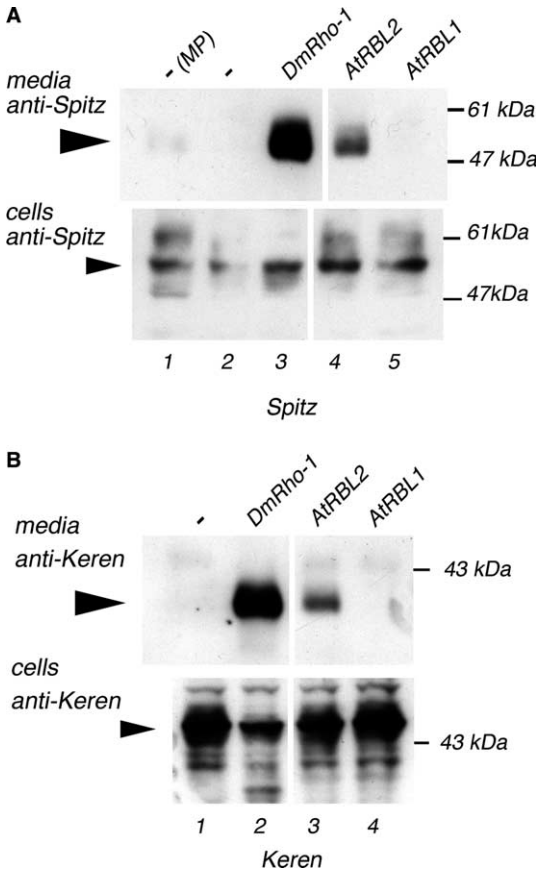

Fig. 4. Proteolytic activity of AtRBL1 and AtRBL2 tested against the two *Drosophila* EGFR ligands. AtRBL1 (A, lane 5; B, lane 4), AtRBL2 (A, lane 4; B, lane 3) and *Drosophila* Rho-1 (A, lane 3; B, lane 2) were tested for their ability to cleave Spitz (A) and Keren (B) in the presence of Star. All experiments except lane 1 of (A) were performed in the presence of 20 μM Batimastat, a potent metalloprotease (MP) inhibitor, to reduce shedding of these ligands by nonspecific cellular enzymes. The cleaved form (large arrowheads) and the uncleaved form (small arrowheads) are denoted for each ligand.

investigation would be required to check the activity of AtRBL1.

In conclusion, the subcellular localization to the Golgi apparatus and the ability to cleave the *Drosophila* EGFR ligands but not TGF indicate that AtRBL2 is indeed a member of Rhomboid intramembrane protease family. Although the overall sequence similarity between Rho-1 and its homologues from other species is not high, amino residues surrounding the catalytic dyad are conserved in AtRBL2 (Fig. 1), suggesting that these motifs might important in recognition of the enzyme substrates.

Although RT-PCR analysis indicated that *AtRBL1* and *AtRBL2* were expressed in all tissues tested (Fig. 2), our initial disruption of either gene alone caused no visible phenotype in plants (data not shown). *Arabidopsis* contains many Rhomboid-like genes, and based on the AtGen Express microarray data (http://jsp.weigelworld.org/atgen/atgen.jsp), all the Rhomboid-like genes are expressed; five Rhomboid-like genes are highly expressed in almost all tissues, while three are limited in some specific tissues. We expect that one or more of the other genes might have overlapping specificity with AtRBL1 while other members of the set might have overlapping specificity with AtRBL2. This has been demonstrated

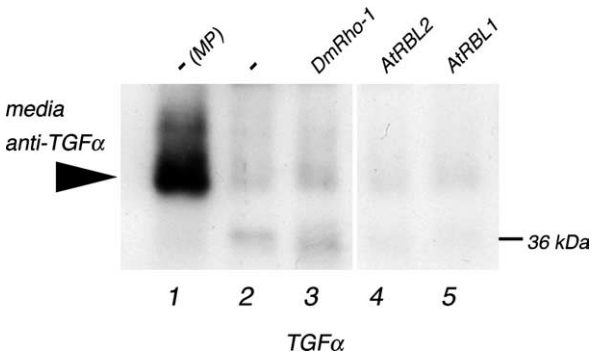

Fig. 5. Substrate specificity of AtRBL1 and AtRBL2 assessed by their ability to cleave the ligand TGFα. AtRBL1 (lane 5), AtRBL2 (lane 4) and *Drosophila* Rho-1 (lane 3) were tested for their ability to cleave TGFα. All experiments were performed in the presence of 20 μM Batimastat, except for the assay depicted in lane 1, where its absence served as a positive control for the expression, trafficking, and proteolytic release of TGFα by cellular metalloproteases (MP).

for *Drosophila* Rhomboid function during development [23,27]. It is also possible that the phenotype may appear only under certain conditions. Further genetic analysis with double or multiple *AtRBL* mutants will be required to assess the biological function of Rhomboid proteins in plants.

Interestingly, plants seem to have no other components of EGF signaling pathway other than Rhomboid. Neither substrates such as Spitz, Keren nor Star can be found by homology search in the genome sequences of either *A. thaliana* or *Oryza sativa*. Formally, plants may have equivalent EGF signaling components that have diverged too much from the proteins found in animals to be detected. However, this is not plausible since the EGF signaling pathway seems to have emerged in the animal lineage after that lineage diverged from plants and fungi. Moreover, Rhomboid proteases recognize a distortion in a short stretch of amino acid in a transmembrane region [28], so the conservation of entire protein is not required for the substrate specificity. Thus, we suggest that AtRBL2 may have a plant specific substrate. Alternatively, since the proteolytic substrates of many animal Rhomboid proteins are not known, the plant enzymes might be targeting an as yet uncharacterized but equally conserved substrates used by these other Rhomboid proteins. The identification of plant Rhomboid substrates might thus provide us with a broader understanding of substrates conserved in other eukaryotes including animals.

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References

[1] Brown, M.S., Ye, J., Rawson, R.B. and Goldstein, J.L. (2000) Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. Cell 100, 391–398.

[2] Weihofen, A. and Martoglio, B. (2003) Intramembrane-cleaving proteases: controlled liberation of proteins and bioactive peptides. Trends Cell Biol. 13, 71–78.
[3] Steiner, H. and Haass, C. (2000) Intramembrane proteolysis by presenilins. Nat. Rev. Mol. Cell Biol. 1, 217–224.
[4] Ye, J., Rawson, R.B., Komuro, R., Chen, X., Dave, U.P., Prywes, R., Brown, M.S. and Goldstein, J.L. (2000) ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. Mol. Cell 6, 1355–1364.
[5] Lemberg, M.K., Bland, F.A., Weihofen, A., Braud, V.M. and Martoglio, B. (2001) Intramembrane proteolysis of signal peptides: an essential step in the generation of HLA-E epitopes. J. Immunol. 167, 6441–6446.
[6] Lee, J.R., Urban, S., Garvey, C.F. and Freeman, M. (2001) Regulated intracellular ligand transport and proteolysis control EGF signal activation in Drosophila. Cell 107, 161–171.
[7] Urban, S., Lee, J.R. and Freeman, M. (2001) *Drosophila* rhomboid-1 defines a family of putative intramembrane serine proteases. Cell 107, 173–182.
[8] Urban, S., Lee, J.R. and Freeman, M. (2002) A family of Rhomboid intramembrane proteases activates all *Drosophila* membrane-tethered EGF ligands. Embo J. 21, 4277–4286.
[9] Urban, S., Schlieper, D. and Freeman, M. (2002) Conservation of intramembrane proteolytic activity and substrate specificity in prokaryotic and eukaryotic rhomboids. Curr. Biol. 12, 1507–1512.
[10] Koonin, E.V., Makarova, K.S., Rogozin, I.B., Davidovic, L., Letellier, M.C. and Pellegrini, L. (2003) The rhomboids: a nearly ubiquitous family of intramembrane serine proteases that probably evolved by multiple ancient horizontal gene transfers. Genome Biol. 4, R19.
[11] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.
[12] An, Y.Q., McDowell, J.M., Huang, S., McKinney, E.C., Chambliss, S. and Meagher, R.B. (1996) Strong, constitutive expression of the *Arabidopsis* ACT2/ACT8 actin subclass in vegetative tissues. Plant J. 10, 107–121.
[13] Kawakami, S. and Watanabe, Y. (1997) Use of green fluorescent protein as a molecular tag of protein movement in vivo. Plant Biotechnol. 14, 127–130.
[14] Uemura, T., Ueda, T., Ohniwa, R.L., Nakano, A., Takeyasu, K. and Sato, M.H. (2004) Systematic analysis of SNARE molecules in *Arabidopsis*: dissection of the post-Golgi network in plant cells. Cell Struct. Funct. 29, 49–65.
[15] Mathur, J. et al. (1998) Gene identification with sequenced T-DNA tags generated by transformation of *Arabidopsis* cell suspension. Plant J. 13, 707–716.
[16] Ueda, T., Yamaguchi, M., Uchimiya, H. and Nakano, A. (2001) Ara6, a plant-unique novel type Rab GTPase, functions in the endocytic pathway of *Arabidopsis thaliana*. Embo J. 20, 4730–4741.
[17] Nagai, T., Ibata, K., Park, E.S., Kubota, M., Mikoshiba, K. and Miyawaki, A. (2002) A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. Nat Biotechnol. 20, 87–90.
[18] The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408, 796–815.
[19] Wasserman, J.D., Urban, S. and Freeman, M. (2000) A family of rhomboid-like genes: *Drosophila* rhomboid-1 and roughoid/rhomboid-3 cooperate to activate EGF receptor signaling. Genes Dev. 14, 1651–1663.
[20] Lemberg, M.K., Menendez, J., Misik, A., Garcia, M., Koth, C.M. and Freeman, M. (2005) Mechanism of intramembrane proteolysis investigated with purified rhomboid proteases. EMBO J. 24, 464–472.
[21] Lohi, O., Urban, S. and Freeman, M. (2004) Diverse substrate recognition mechanisms for rhomboids; thrombomodulin is cleaved by Mammalian rhomboids. Curr. Biol. 14, 236–241.
[22] Boevink, P., Oparka, K., Santa Cruz, S., Martin, B., Betteridge, A. and Hawes, C. (1998) Stacks on tracks: the plant Golgi apparatus traffics on an actin/ER network. Plant J. 15, 441–447.
[23] McQuibban, G.A., Saurya, S. and Freeman, M. (2003) Mitochondrial membrane remodelling regulated by a conserved rhomboid protease. Nature 423, 537–541.
[24] Reich, A. and Shilo, B.Z. (2002) Keren, a new ligand of the *Drosophila* epidermal growth factor receptor, undergoes two modes of cleavage. Embo J. 21, 4287–4296.
[25] Lee, D.C., Rose, T.M., Webb, N.R. and Todaro, G.J. (1985) Cloning and sequence analysis of a cDNA for rat transforming growth factor-alpha. Nature 313, 489–491.
[26] Pascall, J.C. and Brown, K.D. (2004) Intramembrane cleavage of ephrinB3 by the human rhomboid family protease, RHBDL2. Biochem. Biophys. Res. Commun. 317, 244–252.
[27] Urban, S., Brown, G. and Freeman, M. (2004) EGF receptor signalling protects smooth-cuticle cells from apoptosis during *Drosophila* ventral epidermis development. Development 131, 1835–1845.
[28] Urban, S. and Freeman, M. (2003) Substrate specificity of rhomboid intramembrane proteases is governed by helix-breaking residues in the substrate transmembrane domain. Mol. Cell 11, 1425–1434.